

Moreover, both cysteines S549C and S1347C were modified by the larger MTS-biotin (~15Å x 11Å x 8Å) and MTS-rhodamine (~17Å x 16Å x 11Å) but not by MTS-biotin-avidin complex (~80Å x 70Å x 50Å) which, however, could modify wild-type CFTR (likely at C832). These results suggest that, during each gating cycle, NBD1 and NBD2 separate >11Å but <50Å at both composite sites. [NIH-DK51767]

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Structure-Function Analysis of the Anion-Selective Pore of Anoctamin-1

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The anoctamins are a newly described family of anion channels. It has been proposed that anoctamin-1 (Ano1) has 8 transmembrane segments (TMDs) with a re-entrant loop between the 5th and 6th TMD that participates in forming the selectivity filter. To clarify the detailed structure and function of this region, we used cysteine accessibility scanning mutagenesis. Among 16 endogenous cysteines in mAno1, C370, C379, C383, C386, C395, and C836, which are predicted to be in the first and last extracellular loops, are essential for mAno1 function. The construct containing the 6 essential cysteines (mAno16C) was not significantly affected by extracellularly applied, membrane-impermeant MTSET or MTSES. mAno16C was used as a template cysteine scanning mutagenesis of the re-entrant loop (620 - 668). The accessibility of the cysteine-substituted amino acids to MTS reagents were determined as well as the effects on the relative permeability and conductance of the channel to anions. Cysteines introduced at positions 620-626 reduced current amplitude significantly and neither MTSET nor MTSET had significant effects. Cysteine substitution of amino acids 628 - 632 produced currents that were rapidly affected by extracellular MTSET and/or MTSES, suggesting that amino acids 628-632 are near the outer mouth of the channel. Cysteine substitution of amino acids 634 - 662 were not sensitive to MTS reagents although some of these substitutions produced non-functional channels. The ionic selectivity of various mutations in this region were also examined. Further, the accessibility of HA epitopes introduced at various positions near the re-entrant loop was examined to help establish the topology of the channel. These data support the suggestion that amino acids 628-632 may contribute to the outer mouth of the pore, but it remains uncertain whether the re-entrant loop forms the selectivity filter of the channel.

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Volume-Sensitive Chloride Current in Macrophages is Regulated by High-Cholesterol Diet, Cholesteryl Ester Hydrolase and ROS

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Cholesteryl ester (CE) accumulation in macrophages activates proinflammatory mediators and underlies atherosclerotic plaque formation. We tested whether dietary cholesterol regulates volume-sensitive Cl⁻ current (I_{Cl,swell}) via reactive oxygen species (ROS) and determined whether depleting CE by macrophage-specific transgenic over-expression of human cholesteryl ester hydrolase (CEHTg) suppresses I_{Cl,swell}. I_{Cl,swell} was recorded in peritoneal macrophages from LDL receptor-null (Ldlr^{-/-}) and Ldlr^{-/-}CEHTg mice fed chow or a Western-type high-fat, high-cholesterol diet (16 wk). In macrophages from non-fasting Ldlr^{-/-} on chow, I_{Cl,swell} at +60 mV was 6.7 ± 1.4 pA/pF under isosmotic control conditions, increased to 112.6 ± 11.4 pA/pF on exposure to 100-μM H₂O₂ and to 102.9 ± 13.1 pA/pF upon osmotic swelling (0.85T), and was fully blocked by the I_{Cl,swell}-inhibitor DCPIB (10 μM). CEHTg expression did not alter I_{Cl,swell} under control conditions, but significantly reduced I_{Cl,swell} elicited by H₂O₂ to 33.7 ± 5.0 pA/pF and by 0.85T to 24.7 ± 6.3 pA/pF; I_{Cl,swell} remained DCPIB-sensitive. By contrast, I_{Cl,swell} already was strongly activated, 113.9 ± 24.1 pA/pF, under isosmotic control conditions in macrophages from Ldlr^{-/-} fed a high-cholesterol diet; H₂O₂ did not elicit additional current, 126.8 ± 37.7 pA/pF, and DCPIB fully blocked I_{Cl,swell}. High-cholesterol-induced I_{Cl,swell} was ROS-dependent. Both ebelen (15 μM), a ROS scavenger, and rotenone (10 μM), a mitochondrial e⁻ transport blocker, fully suppressed I_{Cl,swell}. Furthermore, CEHTg expression reversed basal I_{Cl,swell} activation by high-cholesterol diet. I_{Cl,swell} in Ldlr^{-/-}CEHTg macrophages was 2.7 ± 0.8 pA/pF under control conditions, was increased to only 27.4 ± 3.7 and 23.1 ± 3.6 pA/pF by H₂O₂ and 0.85T, and was fully blocked by DCPIB. These data suggest that macrophage CE regulate both I_{Cl,swell} under control conditions and its response to stimulation. Fur-

thermore, mitochondrial ROS are required for cholesterol-dependent I_{Cl,swell} activation.

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Facilitators of a Proton-Glutamate in a Cl⁻/H⁺ Exchanger, CLC-EC1

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The CLC-ec1 is a bacterial homologue of the CLC Cl⁻/H⁺ exchanger that catalyzes 2:1 exchange of Cl⁻ and H⁺. Functionally intact monomers form a dimer in bilayer membranes. Gating-glutamate (or Glu_{ex}, E148) is the pH-dependent external gate of both Cl⁻ and H⁺ transport and proton-glutamate (or Glu_{in}, E203) mediates proton-transport from intracellular solution to the protein interior. Mutations on either glutamate with non-protonable amino acids abolish H⁺ transport across the membrane. However, it is still puzzling how protons in the intracellular solution could be transferred to the proton-glutamate forming a salt bridge with arginine (R28) in swapped N-terminal domain from neighboring subunit, which structurally isolates the proton-glutamate from the intracellular solution. In order to find functional facilitators for proton transport, we have been examining several residues near E203. Salt-bridge breaking mutants, R28L, Q and E do not alter the functional activity at all. Moreover, N-terminal truncation mutants (Delta16 and Delta29), which remove an apparent "lid" covering the proton-glutamate, preserve intact Cl⁻/H⁺ exchanger activity. Mutations on two proximate glutamates (E113L and E202L) decrease H⁺ transport rate without changing Cl⁻ turnover rate. Currently, we are investigating functional and structural changes of multiple mutations.

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Specificity of the Regulatory Calcium Binding Site of CLC-K kidney Chloride Channels

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The two human CLC Cl⁻ channels, CLC-Ka and CLC-Kb, are almost exclusively expressed in kidney and inner ear epithelia. Mutations in CLC-Kb and barttin, an essential CLC-K channel β subunit, lead to Bartter syndrome. Previous reports described CLC-K modulation by extracellular calcium and protons. Currents increase with increasing [Ca²⁺]_{ext} and are blocked by increasing [H⁺]_{ext}. Recently we identified a pair of extracellularly accessible acidic residues (E261 and D278) which form an intersubunit Ca²⁺ binding site (Gradogna, Babini, Picollo, Pusch. 2010. J Gen Physiol 136:311). To investigate the specificity of the Ca²⁺ binding site we now have studied the effect of various divalent cations (Zn²⁺, Mg²⁺, Ba²⁺, Sr²⁺, Mn²⁺) on CLC-Ka. Both WT CLC-Ka and the double mutant E261Q/D278N, were blocked by 5 mM Zn²⁺ suggesting that Zn²⁺ affects the channel in a non-specific way. Mg²⁺ does not activate CLC-Ka at concentrations up to 50 mM. In contrast, CLC-Ka was activated by Ba²⁺, Sr²⁺, and Mn²⁺. The rank order of potency was Ca²⁺ > Ba²⁺ > Sr²⁺ = Mn²⁺, likely corresponding to a decreasing affinity of these cations. Furthermore, the Ca²⁺ insensitive double mutant, E261/D278, was also insensitive to Ba²⁺ and Sr²⁺ demonstrating the specificity of the mechanism of activation of CLC-K channels by Ca²⁺.

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Yeast TRK Proteins Mediate Anion Conduction via Barrel-Stave Pores

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Patch-clamp studies of the potassium-transport proteins Trk1,2 in ascomycete fungi have revealed large chloride efflux currents: at clamp voltages negative to -100 mV, with intracellular chloride concentrations > 10 mM (J. Membr. Biol. 198:177, 2004). Current-voltage analysis of these anion currents, especially in *Saccharomyces cerevisiae*, led to an in-series two-barrier model for chloride activation: the lower barrier (designated α) being ~11 kcal/mol and located ~30% into the membrane from the cytoplasmic surface; and the higher one (β) being ~14 kcal/mol and located at or near the outer surface. Quantitative adjustments of this model, most importantly in the amplitude of barrier β, efficiently describe almost all current-voltage data for lyotropic anions, with the order of selectivity being I⁻ ≈ Br⁻ > Cl⁻ > SCN⁻ > NO₃⁻ at pH_o 5.5, and I⁻ ≈ Br⁻ > SCN⁻ > NO₃⁻ > Cl⁻, at pH_o 7.5. The kinetic model evokes a hypothetical structure proposed by Durell & Guy (Biophys. J. 77:789, 1999) on the basis of sequence homology with bacterial potassium channels, plus sequence conservation across fungal species. That model posited an intramembrane